L Number	Hits	Search Text	DB	Time stamp
1	2183	GERARD-$.IN.	USPAT; US-PGPUB; EPO; JPO; DERWENT	2003/08/04 16:55
2	2252	FLYNN-$.IN.	USPAT; US-PGPUB; EPO; JPO; DERWENT	2003/08/04 16:55
3	159	HU-a$.IN.	USPAT; US-PGPUB; EPO; JPO; DERWENT	2003/08/04 16:55
4	1	GERARD-$.IN. and FLYNN-$.IN. and HU-a$.IN.	USPAT; US-PGPUB; EPO; JPO; DERWENT	2003/08/04 16:55
5	4592	GERARD-$.IN. or FLYNN-$.IN. or HU-a$.IN.	USPAT; US-PGPUB; EPO; JPO; DERWENT	2003/08/04 16:55
7	20	RIBOSOM$3 AND (RECOMBINATIONAL ADJ2 CLONING)	USPAT; US-PGPUB; EPO; JPO; DERWENT	2003/08/04 16:56
8	236	(RIBOSOM$3 AND RECOMBIN$9).CLM.	USPAT; US-PGPUB; EPO; JPO; DERWENT	2003/08/04 16:56
9	52	(RIBOSOM$3 WITH RECOMBIN$9).CLM.	USPAT; US-PGPUB; EPO; JPO; DERWENT	2003/08/04 16:56
10	32	(RIBOSOM$3 AND RECOMBINATION$3).CLM.	USPAT; US-PGPUB; EPO; JPO; DERWENT	2003/08/04 16:56
11	2	(RIBOSOM$3 WITH RECOMBINATION$3).CLM.	USPAT; US-PGPUB; EPO; JPO; DERWENT	2003/08/04 16:57
6	1	(GERARD-$.IN. or FLYNN-$.IN. or HU-a$.IN.) AND (RECOMBINATIONAL ADJ2 CLONING)	USPAT; US-PGPUB; EPO; JPO; DERWENT	2003/08/04 16:59
13	427	(life adj2 technolog$4).as.	USPAT; US-PGPUB; EPO; JPO; DERWENT	2003/08/04 17:00
14	188	invitrogen.as.	USPAT; US-PGPUB; EPO; JPO; DERWENT	2003/08/04 17:01
15	575	((life adj2 technolog$4).as.) or invitrogen.as.	USPAT; US-PGPUB; EPO; JPO; DERWENT	2003/08/04 17:01
16	26	(((life adj2 technolog$4).as.) or invitrogen.as.) and (GERARD-$.IN. or FLYNN-$.IN. or HU-a$.IN.)	USPAT; US-PGPUB; EPO; JPO; DERWENT	2003/08/04 17:01

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17	1	(RIBOSOM$3 AND (RECOMBINATIONAL ADJ2 CLONING)) and ((((life adj2 technolog$4).as.) or invitrogen.as.) and (GERARD-$.IN. or FLYNN-$.IN. or HU-a$.IN.))	USPAT; US-PGPUB; EPO; JPO; DERWENT	2003/08/04 17:01
18	17743	(increas$4 or enhanc$4) with recombin$7	USPAT; US-PGPUB; EPO; JPO; DERWENT	2003/08/04 17:03
19	277	((increas$4 or enhanc$4) with recombin$7) with ribosom$4	USPAT; US-PGPUB; EPO; JPO; DERWENT	2003/08/04 17:02
20	417	((increas$4 or enhanc$4) with recombin$7) same (cre or recombinase or int or ihf or hu or flp or xis or hin or cin or gin or resolvase)	USPAT; US-PGPUB; EPO; JPO; DERWENT	2003/08/04 17:05
21	2	(((increas$4 or enhanc$4) with recombin$7) with ribosom$4) same (cre or recombinase or int or ihf or hu or flp or xis or hin or cin or gin or resolvase)	USPAT; US-PGPUB; EPO; JPO; DERWENT	2003/08/04 17:05

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L1        16632 S (FLYNN, ?)/IN,AU
L2        10722 S (GERARD, ?)/IN,AU
L3        86580 S (HU, ?)/IN,AU
L4       113907 S L1 OR L2 OR L3
L5            1 S L1 AND L2 AND L3
L6         3909 S RIBOSOM? (S) RECOMBIN?
L7          479 S L6 (S) (INCREAS? OR ENHANC?)
L8          246 S L6 (S) (STIMULAT?)
L9          654 S L8 OR L7
L10         131 S L9 (S) CLON?
L11        6126 S (BRUCKNER, ?)/IN,AU
L12       56026 S (COX, ?)/IN,AU
L13          97 S L11 AND L12
L14           2 S L13 AND L6
L15           1 DUPLICATE REMOVE L14 (1 DUPLICATE REMOVED)
L16          15 S L10 (S) METHOD?
L17           9 DUPLICATE REMOVE L16 (6 DUPLICATES REMOVED)
L18       46737 S RECOMBIN? (S) (CLONING OR CLONED)
L19        9665 S RECOMBIN? (3N) (CLONING OR CLONED)
L20          49 S L19 (S) RIBOSOM?
L21          47 S L20 NOT L16
L22          35 DUPLICATE REMOVE L21 (12 DUPLICATES REMOVED)

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From:	Leffers, Gerald
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Please forward to me as soon as possible a copy of the following reference:

Bruckner R.C., Cox M.M.; The Histone-like H protein Escherichia coli is ribosomal protein s3. **Nucleic Acids Research 1989, Vol. 17, No. 8, pages 3145-3162.**

Gerald G. Leffers Jr., PhD
Examiner, Art Unit 1636
Crystal Mall 1, Room 11A09
703-308-6232

The histone-like H protein of *Escherichia coli* is ribosomal protein S3

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ABSTRACT

We report the purification of four proteins from Escherichia coli that
stimulate or inhibit inter- and/or intramolecular recombination promoted by
the yeast plasmid-encoded FLP protein. The proteins are identified as the
ribosomal proteins S3 (27 kDa), L2 (26 kDa), S4 (24 kDa), and S5 (16 kDa), on
the basis of N-terminal sequence analysis. The S3 protein is found to be
identical to H protein, an E. coli histone-like protein that is related to
histone H2A immunologically and by virtue of amino acid content. The
H protein/S3 identity is based on co-migration on polyacrylamide gels, heat
stability, amino acid analysis, and effects on FLP-promoted recombination.
These results are relevant to current studies on the structure of the E. coli
nucleoid. Since the H protein has previously been found associated with the
E. coli nucleoid, the results indicate that either (a) some ribosomal
proteins serve a dual function in E. coli, or, more likely, (b) ribosomal
proteins can and are being mis-identified as nucleoid constituents.

INTRODUCTION

The H protein is a histone-like protein derived from Escherichia coli,
originally isolated as an inhibitor of in vitro replication (1). It has been
shown to cross-react immunologically with histone H2A and to have an amino
acid content similar to histone H2A (1). The H protein also has DNA-binding
activity (1,2) and has been identified as one of several proteins associated
with the E. coli nucleoid (3,4). It has been estimated that there are
120,000 copies of H protein in rapidly growing E. coli cells, enough to cover
a significant fraction of the E. coli chromosome (2). This is one of several
proteins playing a significant role in current research to elucidate the
manner in which DNA is packaged in E. coli (3-5). Our interest in this
protein and its function is indirect, the result of an observation occurring
in the course of our studies on site-specific recombination in the yeast
2 micron plasmid.

The 2 micron circle is an autonomously replicating plasmid found in most
strains of yeast Saccharomyces cerevisiae with a copy number of 60 to 100
copies per haploid cell (6,7). The plasmid exists in two isomeric forms in
the cell, which are related by a DNA inversion mediated by a plasmid-encoded
site-specific recombination system (8,9). The recombinase, called FLP
protein, has been purified to homogeneity from E. coli strains in which it
has been expressed (10-13).

Purified FLP protein has several properties that differ from those
observed with partially purified preparations, or that varied unpredictably
from one fraction to the next as purification protocols were developed (12).

Most of these properties involved the overall amount of activity present, and the relative efficiency of intermolecular vs. intramolecular recombination. A purification step could yield either a decrease or increase in total FLP protein activity present. In other cases, different fractions were found that exhibited considerable bias towards intermolecular rather than intramolecular recombination. High concentrations of FLP protein in partially purified preparations were found to inhibit both recombination reactions. In contrast, purified FLP protein exhibits a pronounced and readily explained preference for intramolecular recombination, and inhibition of the reaction is not observed even when the protein is present in large excess (12,13).

Subsequently, an E. coli protein fraction was prepared from E. coli C-600 cells not expressing FLP protein. This fraction duplicated the effects on inter- and intramolecular recombination described above when added to reactions containing pure FLP protein. Low concentrations enhanced the recombination reaction. Large increases in the concentration of the E. coli protein fraction resulted in the inhibition of both reactions (12). These effects were found to be protease sensitive, but were not duplicated by additions of bovine serum albumin (BSA) or other proteins (HU or integration host factor (IHF)) previously shown to affect site-specific recombination in other systems (14,15). Purification of the E. coli protein(s) responsible for these effects was undertaken to determine the nature of the protein(s) involved.

We describe here the purification and characterization of four E. coli proteins that produce the effects on FLP protein mediated recombination described above. One of the proteins has been identified as H protein (1). The N-terminal sequences of the four E. coli proteins were used to search a protein sequence bank to determine if these proteins had been described previously. H protein was found to be identical to the 30S ribosomal protein subunit protein S3 (16). The other proteins correspond to 30S ribosomal subunit proteins S4 (17) and S5 (18), and 50S ribosomal subunit protein L2 (19). All of these ribosomal protein sequences have been confirmed by direct DNA sequencing (20-22). In the course of this study, the focus was shifted from FLP protein to the E. coli nucleoid. Whereas the results begin with effects on FLP-mediated reactions, the primary subject of this report is the H protein - S3 identity and its implications with respect to nucleic acid binding activities in E. coli.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. Bacterial strain C-600 K- was used for the preparation of E. coli proteins (23). Plasmid pMMC3 (10) was purified from the E. coli strain HB101 by banding twice in CsCl gradients in the presence of ethidium bromide (24).

Proteins. FLP protein was purified by C. Gates and its concentration determined as described (13). FLP protein used in experiments was approximately 80% pure. Chicken erythrocyte histone octamers were a gift of Jeff Hanson (Oregon State University). Samples of H protein and HU were a gift of Leroy Bertsch and Arthur Kornberg (Stanford University). IHF was kindly provided by Howard Nash (National Institutes of Health). BamHl was purchased from New England Biolabs. Molecular weight standards for sodium dodecyl sulfate (SDS)/polyacrylamide gel electrophoresis were purchased from Sigma.

Chromatography Resins and Chemicals. Biorex 70, Biogel HTP hydroxylapatite, and Biogel P100 resins were purchased from Biorad. Orange A agarose was purchased from Amicon. Resins were prepared according to the

manufacturer's instructions. Tris was purchased from Boehringer Mannheim Biochemicals. MES, 2-(N-morpholino)ethane sulfonic acid, and TAPS, 3-(N-Tris-(hydroxymethyl)methylamino)-propane sulfonic acid, were purchased from Research Organics. Sodium phosphate monobasic and dibasic was purchased from Mallinckrodt. Ultrapure ammonium sulfate was purchased from Schwarz/Mann Biotech.

Buffers. Buffers of the following composition were used during protein purification procedures. Buffer M is 20 mM MES, pH 6.5, 1 mM ethylenediamine tetraacetic acid (EDTA). Buffer M (2x) contains the same components at twice these concentrations. Buffer P is 20 mM sodium phosphate, pH 6.6, 0.1 mM EDTA. These pH measurements were made at 4°C. Buffer T is 25 mM Tris (80% cation), pH 7.5, 0.5 mM EDTA, pH measured at 25°C. Numbers in brackets following buffer designations, e.g., buffer T [0.1], indicate molar concentration of sodium chloride.

FLP Protein Recombination Assay. The plasmid pMMC3, linearized by digestion with BamHl, which cuts the plasmid once, was used as a substrate in the FLP protein site-specific recombination assay. Plasmid pMMC3 is derived in part from the 2 micron plasmid, and contains two 599 base pair (bp) 2 micron repeat sequences arranged in direct orientation (10). This substrate can undergo either intramolecular or intermolecular recombination when incubated with FLP protein (Fig. 1). Each assay consists of five FLP recombination reactions. Ribosomal or histone proteins were diluted as indicated into 108 μl of reaction buffer. Five 18 μl aliquots were distributed into microtiter wells so that each reaction in a set contains the same amount of ribosomal or histone proteins. The remaining solution was discarded. Control reactions contained no added E. coli or histone proteins. Five serial dilutions of FLP protein were made, and 2 μl of each dilution was added to a reaction. After addition of the indicated amounts of FLP protein, each reaction contained 0.2 μg linear pMMC3 (final concentration of FRT sites = 5.5 nM), 25 mM TAPS buffer, pH 8.0, measured at 25°C, 200 mM NaCl, and 1 mM EDTA, in 20 μl total volume. The reactions were incubated at 30°C for one hour. SDS, 2 μl of 10% (w/v), and 3 μl of gel loading dye (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, and 30% (v/v) glycerol) were added (24). The samples were electrophoresed on an 0.8% agarose gel as described (24). DNA was visualized by staining with ethidium bromide (24).

Determination of Protein Concentration. E. coli protein concentrations were determined by the method of Warburg and Christian (25), and the method of Bradford (26), using bovine serum albumin as a standard.

SDS/Polyacrylamide Gel Electrophoresis. SDS/polyacrylamide gel electrophoresis was performed as described (24). Proteins were visualized by staining with Coomassie blue. Protein molecular weight standards in order of decreasing molecular weight were: bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), E. coli single strand binding protein (18 kDa), and lysozyme (14.3 kDa).

Amino Acid Analysis and N-Terminal Sequencing. Amino acid analysis and N-terminal sequencing were performed by the Protein Sequencing Facility, University of Michigan Medical School, Ann Arbor, Ml. (27). S5 and S4 were sequenced by manual Edman degradation. S3 and L2 were sequenced on an automated system. Protein samples to be analyzed were made 10% (w/v) in trichloroacetic acid and incubated on ice for 30 to 120 minutes. Samples were centrifuged in an eppendorf centrifuge for 10 minutes to collect the precipitate, and the supernatant removed by aspiration. Pellets were washed with acetone and dried under vacuum.

Purification of Ribosomal Proteins (FLP Stimulatory Activities)

Growth of cells. E. coli strain C-600 K- was grown at 37°C in 30 liters of AZ medium (24) in a 50 liter New Brunswick fermenter. During growth the pH was maintained at 7.5 by addition of NaOH. Cells were harvested at A$_{595}$ =

Table 1. Purification of E. coli ribosomal proteins S3, S4, and S5

			Volume (ml)	Protein (mg)
a)				
Fraction	I	Lysis supernatant	238	4300
Fraction	II	Ammonium sulfate ppt.	550	2500
Fraction	III	Biorex-70	36	76
b)		**Purification of S3/H protein**		
Fraction	IVc	Hydroxylapatite	34	15
Fraction	Vb	Biogel P-100	12	6.5
Fraction	VI	Am. sulfate/P-100	9.5	1.3
		Purification of S5		
Fraction	Va	Biogel P-100	12	(4.8*) 0.78
c)		**Purification of S4**		
Fraction	IVb	Hydroxylapatite	28	13
Fraction	V	Biogel P-100	9.5	3.8
Fraction	VI	Orange A agarose	12.5	1.6

Protein concentration of fractions I, II, and Va* determined by the method of
Bradford (26). Protein concentration of the other fractions determined by
the method of Warburg and Christian (25). Letters following fraction numbers
indicate the order of elution from the column.

2.8 (228 g cell paste) and frozen in liquid nitrogen.

Crude Extract. C-600 cell paste, 50 g, was thawed in 100 ml of 50 mM
Tris (50% cation), pH 8.1, measured at 25°C, 10% sucrose (w/v), and 1 mM
EDTA. All purification steps were carried out at 0-5°C. Cells were
disrupted by sonication, on ice, at 120 watts for five minutes, in one minute
bursts. The cells were allowed to cool between bursts. The lysate was
diluted with 100 ml of 25 mM Tris (50% cation), 1 mM EDTA, and 2 M NaCl, and
centrifuged at 18,000 rpm in a Beckmann JA-20 rotor for 80 minutes. The
supernatant, 240 ml, is fraction I (Table 1a).

Ammonium Sulfate Precipitation. Solid ammonium sulfate, 74.6 g (50%
saturation), was added to the lysate gradually over 30 minutes, with
stirring. Stirring was continued for an additional 30 minutes. The
resulting precipitate was collected by centrifugation at 12,000 rpm in a
Beckmann JA-14 rotor for 30 minutes. Both pellets were backwashed with
100 ml of buffer M (2x), 65% saturated in ammonium sulfate (0.43 g/ml) and
cer..rifuged in a JA-14 rotor at 12,000 rpm for 20 minutes. Pellets were
resuspended in 500 ml buffer M [0]. Measured conductivity was equivalent to
buffer M [0.2]. Total volume was 550 ml (fraction II, Table 1a).

Biorex 70 Chromatography. Fraction II was loaded onto a Biorex 70
column (28 x 2.5 cM, 150 ml bed volume) equilibrated in buffer M [0.2], at
1.7 ml/minute. The column was washed with 200 ml buffer M [0.2], and 300 ml
buffer M [0.5]. Some stimulatory activity eluted in the 500 mM NaCl wash.
The bulk of the stimulatory activity was eluted from the column with buffer M
[1.0] (fraction III, 36 ml, Table 1a).

Hydroxylapatite Chromatography. Fraction III, 35 ml, was diluted to a
NaCl concentration of 150 mM by addition of 200 ml of buffer P [0] and
loaded onto a hydroxylapatite column (11.5 x 1.5 cM, 20 ml bed volume)
equilibrated with buffer P [0.15]. The column was washed with 20 ml of
buffer P [0.15]. Protein was eluted with a 400 ml linear gradient from

150 mM NaCl to 1.0 M NaCl, in buffer P. The column was run at 0.6 ml/minute and 3 ml aliquots were collected in plastic test tubes. Three sets of fractions were pooled (fraction IVa; not shown, IVb (Table 1c), and IVc (Table 1b). The letters following a fraction number refer to the order of elution from the column. Table numbers refer to the purification table where the data appears. Fraction IVa, tubes 28-34, contained L2. L2 degraded during short-term storage on ice. For this reason, L2 was generally purified in a separate preparation as described below. Fraction IVb, tubes 50-58, contained S4. Fraction IVc, tubes 60-70, contained S3/H protein, and S5. Some stimulatory activity was present at other points in the gradient. It was not determined whether this was due to the presence of small amounts of the proteins described here, or to additional proteins that remain unidentified.

Purification of S3/H Protein and S5

Biogel P100 Chromatography. Fraction IVc, 34 ml (Table 1b), was dialyzed against two liters of buffer T [0.1]. To concentrate this fraction, it was loaded onto a 1 ml Biorex 70 column equilibrated in buffer T [0.1]. The column was washed with 1 ml of buffer T [0.1], and the protein eluted with buffer T [1.0]. The protein containing eluate was collected in 1.6 ml. This was applied to a Biogel P100 gel filtration column (47 x 3 cM, 225 ml bed volume) equilibrated in buffer T [0.05]. The column was run at 10 ml/hour, and 2.5 ml aliquots were collected. S5 eluted just after the void volume had passed through the column, tubes 33-37. These tubes were pooled to give fraction Va, 12 ml. Densitometric scanning of a Coomassie blue stained SDS/polyacrylamide gel of fraction Va indicated that S5 accounted for greater than 98% of the stained material (Table 1b, Fig. 2a).

Tubes 45-49, which contained S3/H protein, were also pooled to give fraction Vb (Table 1b). Approximately 80% of the protein in this fraction was S3/H protein. Most of the remainder was S4. The protein peak of S4 appeared just after the protein peak of H protein on the Biogel P100 column. In addition, a small amount of S4 appeared after the column void volume, and trailed throughout the column. These properties allowed separation of the two proteins by repetition of the same Biogel P100 column. A portion of fraction V (8 ml, 4 mg) was precipitated by addition of solid ammonium sulfate, 4.5 g (80% saturation), and stirred for 30 minutes. The precipitate was collected by centrifugation at 18,000 rpm in a Beckmann JA-20 rotor. The pellet was resuspended in 1 ml of buffer T [0.05], and applied to the 225 ml Biogel P100 column. The column was run at 10 ml/hour and 2.5 ml aliquots were collected. Tubes 44-47 contained S3/H protein greater than 95% pure (fraction VI, Table 1b) (Fig. 2a).

Purification of S4

Biogel P100 Chromatography. Fraction IVb, 28 ml (Table 1c), was dialyzed against two liters of buffer T [0.1] and loaded onto a 1 ml Biorex 70 column equilibrated in buffer T [0.1] to concentrate it. The column was washed with 1 ml buffer T [0.1]. Protein was eluted from the column with buffer T [1.0]. The protein was collected in a total volume of 1.2 ml, and was applied to the same 225 ml bed volume Biogel P100 column equilibrated in buffer T [0.05]. The column was run at 10 ml/hour and 2.5 ml aliquots were collected. Tubes 51-54 were pooled to give fraction V (Table 1c).

Orange A Agarose Chromatography. Fraction V was loaded onto a 3 ml Orange A agarose column equilibrated in buffer T [0.05]. The column was washed with 3 ml buffer T [0.05]. Protein was eluted with a 60 ml linear gradient from 50 mM NaCl to 1 M NaCl in Buffer T. The column was run at 0.2 ml/minute and 1.5 ml aliquots were collected. Tubes 22-29 were pooled to give fraction VI, 9.5 ml. This fraction contained S4 that was greater than 95% pure (Table 1c, Fig. 2b).

Table 2. Purification of ribosomal protein L2

		Volume (ml)	Protein (mg)
Fraction I	Lysis supernatant	238	4300
Fraction II	Ammonium sulfate ppt.	550	2800
Fraction III	Biorex-70	44	88
Fraction IVa	Hydroxylapatite	5.0	4.3
Fraction V	Biogel P-100	7.5	0.48

Protein concentrations of fractions I and II determined by the method of Bradford (26).   Protein concentrations of fractions III, IVa, and V determined by the method of Warburg and Christian (25).

Purification of L2

Hydroxylapatite Chromatography. L2 was purified from a separate batch of cells.   Fraction III, 43 ml (Table 2), was prepared from 50 g of E. coli C-600 cells as described above.   Fraction III was diluted to 150 mM NaCl by addition of 215 ml of buffer P [0], and loaded onto a hydroxylapatite column (9 x 1.5 cM, 15 ml bed volume) equilibrated with buffer P [0.15].   The column was washed with 15 ml of buffer P [0.15].   Protein was eluted with a 200 ml linear gradient from 150 mM NaCl to 1 M NaCl in buffer P.   The column was run at 0.9 ml/minute and 2.5 ml aliquots were collected.   Tubes 23 and 24 were pooled to give fraction IVa, 5 ml (Table 2).

Biogel P100 Chromatography.   Fraction IV was precipitated with ammonium sulfate (80% saturation).   The pellet was resuspended in 600 µl buffer T [0.05] and applied to a 225 ml Biogel P100 column equilibrated in buffer T [0.05].   The column was run at 10 ml/hour and 2.5 ml aliquots were collected. Tubes 49-51 contained L2 that was greater than 95% pure (fraction V, Table 2, Fig. 3).

RESULTS
Notes on the Purification Procedures

The initial goal of these experiments was to purify the protein or proteins that stimulated the recombinase activity of FLP protein in vitro. The FLP recombination assay is illustrated in Figure 1. The E. coli protein activity is made evident by an increase in the number and intensity of DNA bands resulting from intermolecular recombination compared with control reactions containing only FLP protein.

In preliminary trials, a number of purified proteins were added to the in vitro FLP recombination reaction, in an attempt to duplicate the stimulatory effect and possibly identify the factor responsible. Bovine serum albumin (1 mg/ml), which might stabilize the dilute FLP protein, had no effect. No stimulation was detected when protein HU, or integration host factor (IHF), was added to the FLP recombination reaction. Both of these proteins have been shown to have an effect on site-specific recombination in other systems (14,15).   Other factors that were tested, the highly basic protein lysozyme, E. coli topoisomerase I, E. coli single strand binding protein, and spermidine, all failed to stimulate FLP recombination. These efforts made it clear that identification of the responsible E. coli activities would require their purification.

During early fractionation attempts, activity was found to elute across a wide range of salt concentrations when gradients were run on a number of chromatographic resins, and across a wide molecular weight range when gel
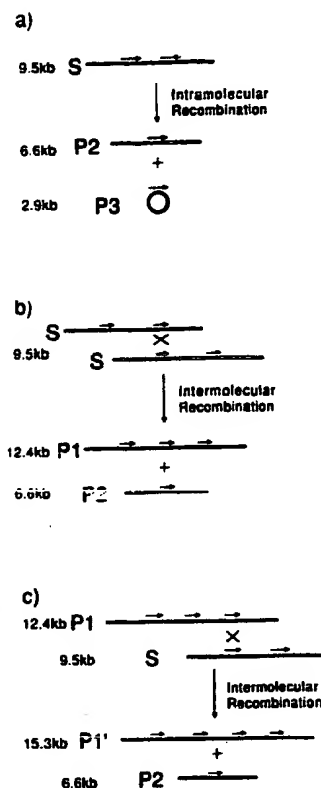
Figure 1. __FLP recombination assay__. The assay of FLP protein-mediated site-specific recombination uses a 9.5 kb linear DNA substrate molecule, S, which contains two FLP recombination sites arranged in direct orientation. Two types of recombination reactions can be observed: a) Intramolecular recombination produces two products, P3, a 2.9 kb circular DNA molecule, and P2, a 6.6 kb linear DNA molecule. b) Intermolecular recombination between two different FLP recombination sites produces a 6.6 kb linear product, P2, identical to that produced in the intramolecular reaction, and a 12.4 kb linear product, P1. c) P1 can react further with S, to produce larger products. These can be seen as a ladder of bands (Figs. 4 and 5), each larger in size by 2.9 kb.

filtration columns were run. This suggested that multiple active species were present, and a decision was made to purify more than one. In several cases, separation methods were developed by following the appropriate protein band in SDS/polyacrylamide gels, and pooling those fractions that contained the desired protein. These pools were then assayed to ensure that they contained stimulatory activity. Activity assays were not usually performed until fraction III (Biorex 70), since stimulation of FLP protein recombination was difficult to detect in earlier fractions.

Several of the different active protein fractions generated in the early work had only two protein bands in common, both in the 25-27 kDa molecular weight range on an SDS/polyacrylamide gel. The size (27 kDa) and heat stability (see below) suggested that H protein might be the stimulatory factor in these fractions (1). A sample of H protein was obtained (see
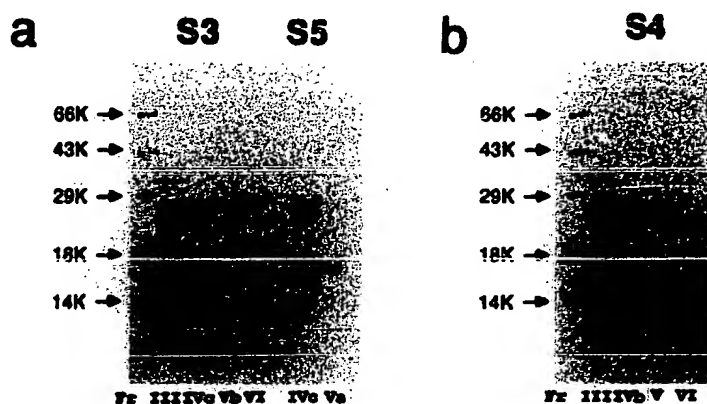
Figure 2. <u>Purification of ribosomal proteins</u>. Roman numerals refer to the fractions listed in Table 4.1. a) Protein fractions generated during purification of S3/H protein and S5. b) Protein fractions generated during purification of S4. 12% running gel, 6% stacking gel.
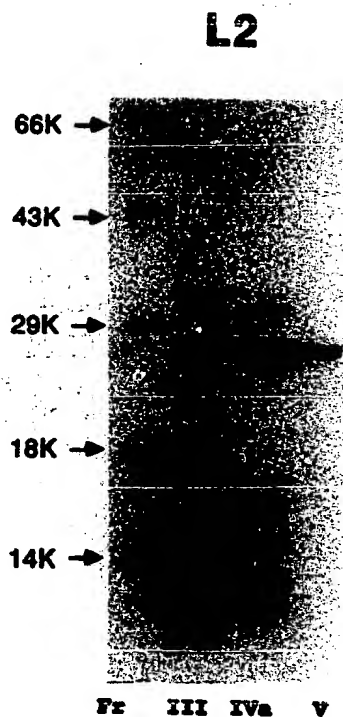

Figure 3. <u>Purification of ribosomal protein L2</u>. Roman numerals refer to the fractions listed in Table 4.2. Protein fractions were generated during the purification of L2. 12% running gel, 6% stacking gel.

Table 3. Amino acid composition of purified ribosomal proteins

				Amino acid composition, mol%			
	L2	S5	S4	S3/H protein	H protein (1)	S3 (16)	H2A (28)
Ala	9.7	11.8	8.7	10.7	11.3	10.3	12.9
Asx	8.8	7.4	7.3	6.9	7.6	6.7	6.6
Glx	6.8	9.0	12.1	10.3	11.6	10.8	9.8
Phe	1.5	2.6	2.1	2.3	1.3	2.2	0.9
Gly	12.4	12.9	8.1	9.0	9.7	8.2	10.8
His	3.0	2.8	1.8	2.1	1.7	1.7	3.1
Ile	4.6	5.0	3.4	6.8	6.5	7.8	3.9
Lys	9.1	7.8	10.4	10.4	10.7	9.9	10.2
Leu	7.6	6.0	10.1	6.1	7.2	5.6	12.4
Met	1.6	3.8	1.7	1.6	0	1.7	0
Pro	5.8	3.6	3.5	4.8	4.8	4.3	4.1
Arg	11.3	6.8	11.7	10.4	9.9	9.1	9.4
Ser	3.3	3.6	5.2	3.7	4.3	3.9	3.4
Thr	3.9	4.9	3.4	4.7	4.5	4.7	3.9
Val	8.3	10.6	7.1	8.6	8.3	9.5	6.3
Tyr	2.6	1.3	3.4	1.9	0.4	1.7	2.2
MW	26,018	17,001	25,018	26,980	28,000	25,852	14,000
Residues	241	162	223	247	-	232	-
Lys/Arg	0.81	1.15	0.89	1.00	1.08	1.09	1.09
Gly X Arg	140	88	95	94	96	90	102

Table 4.   N-terminal sequences of the purified ribosomal proteins

S5	AHIEKQAGEL
	AHIEKQAGEL
S4	AHYLGPKLKL
	ARYLGPKLKL
S3	GQKVHPNGIR-LGIVKPWNST-WFANTKEFAD-NLDSDFKVRQ-YLT
	GQKVHPNGIR-LGIVKPWNST-WFANTKEFAD-NLDSDFKVRQ-YLT
L2	AVVKLKPTSP-GRRKPVVVVN
	AVVKCKPTSP-GRRHVVKVVN

N-terminal sequences for the purified ribosomal proteins are listed on the
top line.   Published N-terminal sequences are listed on the bottom line.
Positions where the sequences are different are underlined.   References: S3
(16,20), S4 (17,21), S5 (18,22), L2 (19,20).

Methods) for comparison.   The donated preparation contained one major
contaminant (26 kDa), as is the case with some preparations of H protein
generated by published methods.   This material helped us to identify
H protein and the single contaminant as two of the proteins responsible for
the effects on FLP protein activity as described below.   A 24 kDa protein and
a 16 kDa protein were purified from additional fractions that were found to
stimulate FLP protein recombination.   These proteins have been identified as
described below.   H protein is identical to 30S ribosomal subunit protein S3
(16,20).   The 16 kDa protein and the 24 kDa protein correspond to S5 (18,22)
and S4 (17,21), respectively.   The 26 kDa protein is L2, of the 50S ribosomal
subunit (19,20).   For convenience, the ribosomal protein designations are
used throughout.   The evidence to support these identifications is presented
below.   Protein S3 is referred to as S3/H protein, to facilitate discussion
and comparison with published data on H protein.

Identification and Properties of Purified Ribosomal Proteins.   Molecular
weights of the E. coli protein monomers, determined by comparison of the
migration on an SDS/polyacrylamide gel with the migration of molecular weight
standards, were 27, 26, 24, and 16 kDa (Figs. 2 and 3).   Amino acid
compositions and N-terminal sequences are listed in Tables 3 and 4.   These
four proteins were identified as ribosomal proteins S3, L2, S4, and S5 (in
order of decreasing size) on the basis of N-terminal sequences.   Published
N-terminal sequences of these proteins are presented for comparison.
Protein S3 was further identified as H protein by several   criteria:
1) comigration on a polyacrylamide gel with a sample of H protein obtained
from the Kornberg laboratory (not shown, note that protein L2 comigrated with
the major H protein contaminant);   2) heat stability (see below);
3) stimulation of FLP protein-promoted recombination similar to the
stimulation observed with the donated H protein preparation; and 4) amino
acid analysis (Table 3).   The previously reported amino acid compositions of
S3 (16) and H protein (1) are presented in Table 3 for comparison.   Both are
in excellent agreement with our results.   The observed N-terminal sequences
of S3/H protein (Table 4) match the published sequence of S3 (16,20) to the
extent it was determined (to position 43).

The N-terminal sequences of the four proteins show variations from
published data in two cases.   Our sequence for S4 has a histidine at
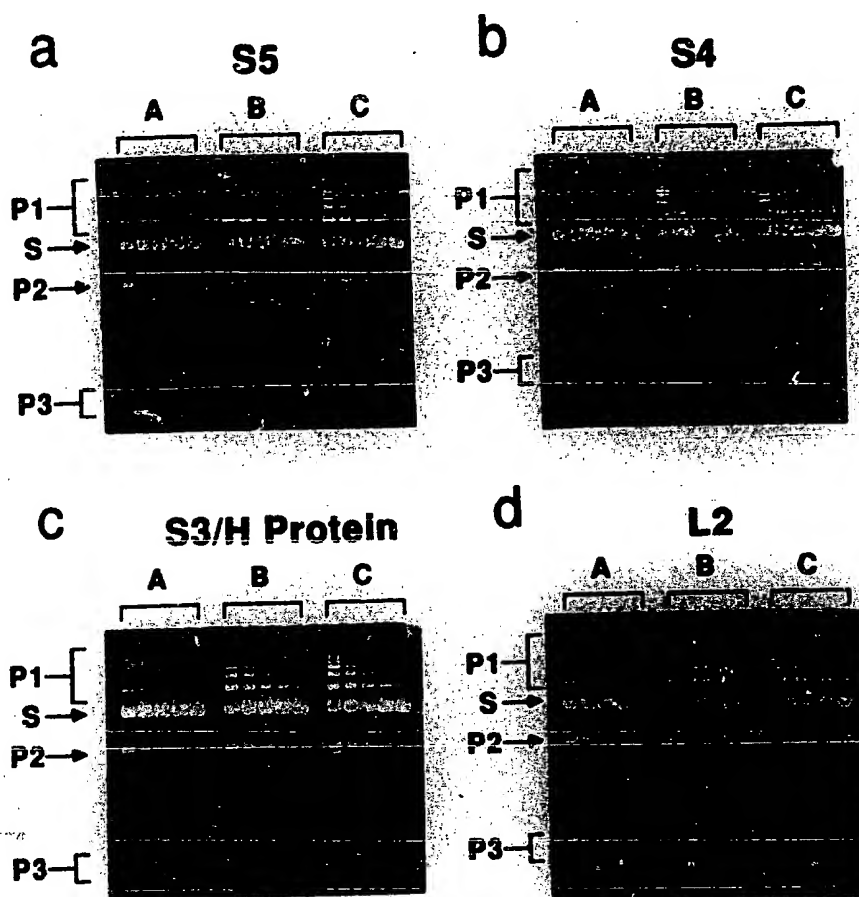position 2, compared with an arginine in the published sequence (17,21).   The

Figure 4. <u>Stimulation of FLP protein-mediated site-specific recombination by purified ribosomal proteins</u>. Each set of reactions consists of five dilutions of FLP protein. From left to right in each set 60, 30, 15, 7.5, or 3 0 pg of FLP protein are added. Three sets of reactions are shown for each purified ribosomal protein: a) Control, no added ribosomal protein. b) Ribosomal protein is added to each reaction at a level that produces a stimulation of intermolecular recombination. This results in an increase in the products, P1. c) Ribosomal protein is added to each reaction at a level that increases the amount of intermolecular recombination, while inhibiting the intramolecular recombination reaction. This results in an increase in the products, P1, and a decrease in P3. The following amounts of ribosomal proteins were added to each reaction:
a) Set B, 0.42 μg S5;   set C, 0.84 μg S5;
b) Set B, 0.26 μg S4;   set C, 0.52 μg S4;
c) Set B, 0.42 μg S3/H protein; set C, 0.84 μg S3/H protein;
d) Set B, 0.15 μg L2;   set C, 0.40 μg L2.

sequence of L2 matches the published sequence at only 16 of 20 positions. We do not know whether the discrepancy is due to a sequencing error in our data. The possibility exists that this is a new protein with homology to L2 at the N-terminus.
    H protein (S3) was shown to be a dimer in solution at low NaCl
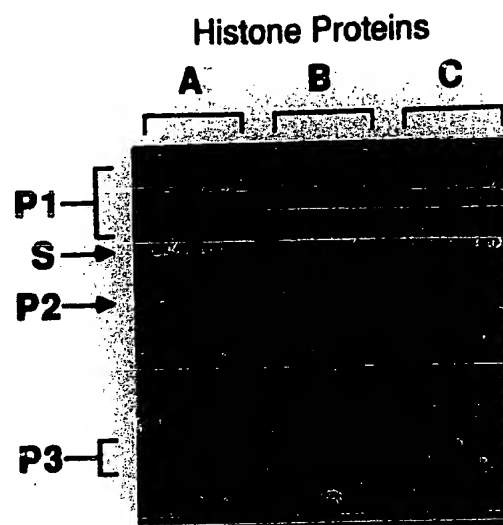
Histone Proteins

Figure 5. <u>Stimulation of FLP recombination by histone proteins</u>. These experiments are identical to those illustrated in Figure 4, except that histone proteins (chicken erythrocyte) were added to set B and set C. Set A, control reactions, no added histone protein; set B, 0.55 µg; set C, 0.77 µg, added to each reaction.

concentrations (1). In the present study S3/H protein, L2, and S4 eluted in order from a Biogel P100 column at positions expected if these proteins form stable homodimers (not shown). Some of the S4 and all of the S5 were found in the void volume, suggesting the formation of higher order aggregates. Small amounts of S4 trailed throughout the column. Earlier work had indicated that these proteins were elongated or cylindrical in shape (29). These proteins are now believed to be globular (30). Deviations from a globular structure would affect the elution of a protein from a sizing column.

The purified ribosomal proteins were heated to 90°C for five minutes, then centrifuged to remove any precipitate. The proteins were assayed for FLP protein stimulatory activity, as described below, along with identical samples that had not been heated. S3/H protein, L2, and S5 showed little or no loss of stimulatory activity after heating. S4 lost approximately half of its activity, but was still able to stimulate FLP protein recombination.

<u>Effect of Ribosomal Proteins on FLP Protein Mediated Site-specific Recombination</u>. The linear DNA substrate pMMC3 (S) used in the FLP protein recombination assay can undergo intramolecular recombination (Fig. 1a), to produce a circular product, P3, and a smaller linear product, P2. pMMC3 can also undergo intermolecular recombination (Fig. 1b), to produce a larger linear product, P1, and a smaller linear product identical to that produced by intramolecular recombination, P2. P1 can react further with substrate (Fig. 1c), to produce a series of larger linear products, which appear on an agarose gel as a ladder of bands migrating above the substrate band (31). Products collectively designated P1 are diagnostic of an intermolecular recombination reaction, while the product designated P3 is diagnostic of an intramolecular recombination reaction.

The effect of the purified ribosomal proteins on FLP protein mediated

site-specific recombination is shown in Figure 4. Three sets of FLP protein recombination reactions are shown to illustrate the effect of each of the four purified ribosomal proteins. Each of the four ribosomal proteins has a similar effect on FLP protein recombination. Each reaction in a set contains a consecutively decreasing amount of FLP protein (from left to right, see Methods). The ratio of FLP protein monomer to FLP recombination site in each consecutive reaction is 22:1, 11:1, 5.5:1, 2.8:1, and 1.1:1.

The sets of reactions labeled A are control reactions and contain no added ribosomal protein. The sets of reactions labeled B and C contain added ribosomal protein, and each reaction within a set contains the same amount of added protein. In the sets of reactions labeled B, an amount of ribosomal protein (determined empirically, see Table 5) sufficient to stimulate the production of intermolecular recombination products, P1, compared to the control set of reactions in set A, is added to each reaction. Intramolecular recombination products, P3, are also observed, and in some cases, they are more intense than in the control reactions (Fig. 4a,b, and d).

In the sets of reactions labeled C, the amount of ribosomal protein added to each reaction was doubled relative to set B. An exception was the experiments with L2, where 2.6 times the amount of protein in B was added to C. Production of intermolecular recombination products, P1, is increased further, while production of intramolecular recombination products, P3, is inhibited.

H protein was shown to inhibit the activity of a number of DNA binding proteins (12). This inhibition required a stoichiometric amount of H protein, with saturation reached at a ratio of one H protein dimer/75 bp of DNA. Using this value, there are 125 H protein dimer binding sites/pMMC3 molecule. The amount of H protein in the H protein assays corresponds to 20% (set B) and 40% (set C) saturation of the DNA substrate.

The ratios of ribosomal protein monomers/pMMC3 molecules in the sets of reactions shown in Figure 4 are shown in Table 5. The higher ratios of S5 required for stimulation may reflect its higher aggregation state. There are enough ribosomal protein monomers in each experiment to partially coat each DNA molecule. We do not know whether the ribosomal proteins affect primarily the DNA or the FLP protein.

We have not yet developed a reliable quantitative measurement of the stimulation of the FLP protein recombination reaction. The assays shown give a qualitative measurement of the stimulation based on the number and intensity of intermolecular recombination product bands observed for each reaction, and the amount of FLP protein needed to observe a detectable recombination reaction, compared to control reactions (set A) where no ribosomal protein is added. The number and intensity of FLP protein intermolecular recombination products are greatly increased by addition of ribosomal proteins. As many as six intermolecular recombination product bands can be observed in sets B and C for the highest concentration of FLP protein vs. the control set, A, where only one is seen. In set C, recombination products can be detected at a five fold lower concentration of FLP protein (15 ng FLP protein/reaction vs. 3 ng FLP protein/reaction). In some cases the intramolecular recombination products in set B are more intense than in the control reactions, and can be observed at lower FLP protein concentrations (Fig. 4a,b, and d). This indicates that the ribosomal proteins do not increase intermolecular recombination solely by decreasing intramolecular recombination.

In the sets of reactions labeled C, the intramolecular recombination reaction is inhibited while the intermolecular recombination reaction is stimulated further. The purified ribosomal proteins were too dilute to allow addition of enough protein to the FLP recombination reaction to cause

Table 5. Ratios of ribosomal protein monomers:pMMC3 DNA

	Set B	Set C
S3/H protein	53	106
S4	37	74
S5	140	280
L2	19	51

The ratios of ribosomal protein monomers:pMMC3 in the sets of reactions in Figure 4 are listed.

inhibition of all reactions. S5 was concentrated and titrated into the FLP recombination reaction (not shown). The amount of FLP protein added was the same in each reaction. The number of intermolecular recombination products increased at first, and then leveled off, followed by a significant drop in the number and intensity of products. Complete inhibition was not achieved. In assays where mixtures of the ribosomal proteins were present, complete inhibition was observed at high concentrations (not shown). It is possible that these proteins could have a synergistic effect.

Effect of Histone Proteins on FLP Protein Mediated Site-specific Recombination. H protein has an amino acid composition similar to histone protein H2A (Table 3), and reacts with antibodies made against H2A (1). The stimulatory effect of H protein suggested that histone proteins (32) might also stimulate FLP protein recombination.

Intact chicken erythrocyte histone octamers (33) in 2 M NaCl (no histone H1 was present in this preparation) were diluted to 1 M NaCl, and then to 200 mM NaCl in the FLP protein recombination reaction mixture. Dilution into low salt causes the histone octamers to dissociate into an H3-H4 tetramer, and two H2A-H2B dimers (34). The effect of histone proteins on FLP protein site-specific recombination is shown in Figure 5.

The effect of histone proteins on FLP protein recombination is similar to that of the ribosomal proteins, although the degree of stimulation is not as great. In the set of reactions labeled B, the intermolecular recombination is stimulated relative to the control reactions in set A, while the intramolecular recombination products, P3, are still observed. In the set of reactions labeled C, the intermolecular recombination reaction is stimulated further, while the intramolecular recombination reaction is inhibited. FLP protein recombination products can be detected at a five fold lower concentration of FLP protein in set C, compared to the control reactions. Increasing the concentration of the histone proteins leads to complete inhibition of the FLP protein recombination reaction.

The species responsible for the stimulation has not been determined. Both the H2A-H2B dimer and the H3-H4 tetramer could cause the stimulation. Alternatively, if only one of these species is active, the other could bind DNA as a competitive inhibitor. This would lower the effective concentration of the active species on the DNA and account for the lower level of stimulation observed, compared to the stimulation of FLP protein recombination caused by the ribosomal proteins.

DISCUSSION

The primary finding of this study is that H protein is ribosomal protein S3. This protein has been linked to the E. coli nucleoid by virtue

of its histone-like properties (1), its DNA binding activity (1), the large amount of it in the cell (2), and its presence in nucleoid preparations (4). At least three other ribosomal proteins (S4, S5, L2) appear to have similar properties. All of these proteins mimic histones at least with respect to some physical properties and effects on FLP-mediated recombination. This observation is important to current efforts to elucidate the structure of the E. coli nucleoid. Either some ribosomal proteins serve a second function - DNA packaging - in E. coli, or, more likely, some ribosomal proteins are fortuitously associated with the nucleoid in many preparations.

Association of some ribosomal proteins with DNA should not be surprising. All four of the proteins identified here bind to rRNA (35). The DNA binding activity of S3/H protein (1), and evidently some other ribosomal proteins, is most likely a simple manifestation of their function in ribosome structure. Preparation of crude extracts in the presence of EDTA tends to dissociate ribosomes, making these proteins the major basic, low molecular weight contaminants in such a preparation. As indicated by the published properties of H protein, these proteins can affect many processes in DNA metabolism in vitro.

We have considered the possibility some or all of these ribosomal proteins serve a second function in DNA packaging. The link is strongest with the H protein (1,2). This protein binds to duplex DNA and catalyzes re-annealing of complementary single strands, properties associated with histones (1). It is also very heat-stable and exhibits an evident structural identity with histone H2A (1). A scan of sequence databases reveals there is significant homology on the amino acid level between H2A and E. coli S3 with 20% or greater amino acid identity in some extended regions depending on alignment and the species from which the H2A sequence is taken. While this may suggest a basis for the immunological cross-reactivity of S3 and H2A, much of the homology involves regions highly enriched for glycine, alanine, arginine and lysine (many of the amino acid matches involve these residues) and may be a fortuitous reflection of the nucleic-acid binding function of both proteins. Interestingly, both polypeptides of the best characterized E. coli histone-like protein, HU, exhibit significant amino acid sequence homology with ribosomal protein S5 in a wide range of bacterial species (36,37). Nevertheless, the links are generally circumstantial and the results are best explained by a fortuitous DNA binding activity of these ribosomal proteins. EDTA is commonly added to cell extracts in an effort to reduce the activity of enzymes such as nucleases. Since EDTA facilitates the dissociation of ribosomes, ribosomal proteins are present in abundance in such extracts. The potential for artifact production, especially in studies of DNA metabolism and DNA packaging, is obvious.

The effects of these ribosomal proteins on FLP-mediated recombination explain several published observations made with partially purified FLP protein fractions (31,38). An apparent bias toward intermolecular recombination seen in some studies (31) was clearly due to the effects of these contaminants. The results almost certainly do not reflect any specific effect of these proteins on the FLP system. Instead, they provide new information about the complex interrelationships and roles of nucleic acid binding proteins in E. coli.

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